AD_____

Award Number: DAMD17-98-1-8175

TITLE: Inhibiting Tumorigenesis by Growth Factor Receptor Down Regulation Using a Sorting Nexin

PRINCIPAL INVESTIGATOR: Richard Kurten, Ph.D.

CONTRACTING ORGANIZATION: University of Arkansas
for Medical Sciences
Little Rock, Arkansas 72205-7199

REPORT DATE: April 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Annual (15 Apr 00 - 14 Mar 01)

4. TITLE AND SUBTITLE
Inhibiting Tumorigenesis by Growth Factor Receptor Down Regulation Using a Sorting Nexin

5. FUNDING NUMBERS
DAMD17-98-1-8175

6. AUTHOR(S)
Richard Kurten, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72205-7199

E-Mail: KurtenRichardC@uams.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Excessive activation of growth factor receptors can lead to the unrestrained cellular proliferation characteristic of tumors. Our objective is to determine if SNX1, a protein involved in intracellular membrane trafficking, can be used to downregulate EGF receptors in mammary gland. Our approach is to characterize the gene for SNX1 and to generate transgenic animals overexpressing SNX1 in mammary glands. We have characterized a genomic clone for SNX1 and had planned to use this clone for transgenic vector construction. However, the size of the first intron in SNX1 wass too large for this approach to be used successfully. Instead, a WAP-SNX1 cDNA vector was been constructed and was used to generate transgenic animals. One transgen positive mouse has been identified.

A Career Development Award was a second component of the application. Career development activities include: participation as reviewer on the American Cancer Society Cell Structure and Metastasis study section and participation on a search committee charged with identifying a Director for Breast Cancer Research at UAMS. In addition, the State of Arkansas Breast Cancer Research Program awarded me a one-year pilot research grant to examine the relationship between HER-2/*neu* and EGF receptors in mammary gland cell proliferation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, down regulation, sorting nexin, transgenic, EGF receptor, TGFα	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

May 14, 2001

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Inhibiting Tumorigenesis by Growth Factor Receptor Down Regulation Using a Sorting Nexin

The development of the mammary gland and its normal function in lactation is controlled by a variety of steroid hormones and peptide growth factors whose concentrations vary depending on the functional status of the gland. Disruption of this complex signaling network by genetic damage, environmental toxins or other factors can override normal restraints on cell growth and lead to the formation of tumors. Epidermal growth factor (EGF) levels been found to be elevated in breast tumor tissues and are an indicator of poor survivability because these tumors are generally resistant to therapies aimed at interruption of estrogen action. As a consequence of elevated EGF receptor levels, proliferative responses to growth factors are enhanced. This can result in hyperplasia and an increased probability of mutations occurring that further contribute to unrestrained tumor growth.

To prevent excessive mitogenic signaling, ligand bound receptors are removed from the cell surface in clathrin coated pits. Once inside the cell, receptor-ligand complexes are sorted away from nutritional receptors like the LDL receptor and targeted for degradation in lysosomes. The membrane trafficking events underlying lysosomal targeting involve the recognition of small amino acid "codes" by the sorting machinery. I recently discovered a protein, the sorting nexin, that recognizes the EGF receptor targeting code and stimulates its transport to the lysosome. In tissue culture cells, overexpression of sorting nexin 1 (SNX1) down regulated EGF receptors by dramatically shortening receptor half-life. As a consequence, EGF-stimulated receptor tyrosine kinase and immediate early gene activities were inhibited substantially. To test the hypothesis that SNX1 can be used *in vivo* to regulate mitogenic signaling by down regulating the EGF receptor, thereby inhibiting tumorigenesis, I propose to use an established transgenic mouse model for therapeutic intervention. In transgenic mice engineered to overexpress transforming growth factor alpha (TGFα), a ligand for the EGF receptor, mammary gland tumors invariably occur in females after 2 or 3 pregnancies. To determine if it is feasible to inhibit TGFα induced mammary gland tumorigenesis by overexpressing SNX1, the following technical objectives are proposed:

1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA.
2. Prepare and characterize a transgenic mouse line with SNX1 expression targeted to the mammary gland using the whey acidic protein (WAP) promoter.
3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

BODY

Technical Objective 1. Isolate and characterize the human SNX1 gene using the SNX1 cDNA that I cloned.

Task 1: **Months 1-2: Plate and screen genomic phage library by hybridization with ^{32}P labeled SNX1 cDNA. Prepare plaque pure phage stocks.**

Progress: In collaboration with H. Steven Wiley, University of Utah School of Medicine, 3 bacterial artificial chromosomes (BAC) containing the human SNX1 gene were isolated. Fluorescence *in situ* hybridization was used to determine the chromosomal localization of both SNX1 and SNX2 (Figure 1). Three different P1 clones were used to deduce the chromosomal localization of SNX1 and 2 were used for SNX2. Hybridization efficiency ranged from 85 to 91%. SNX1 was localized to human chromosome 15q22 and SNX2 was localized to chromosome 5q23.

Task 2: **Months 3-4: Purify phage DNAs and characterize the phage inserts by restriction mapping and Southern hybridization**

Progress: One of the BAC clones for SNX1, labeled 6K1, was characterized by subcloning and restriction mapping (Figure 2). Characterization of multiple clones was not practical due to the unexpectedly large size of the SNX1 gene.

Task 3: **Months 5-6: Subclone phage inserts that appear to contain the entire SNX1 coding region; confirm that they do by sequencing using primers complimentary to the ends of the SNX1 coding region and select the largest one for further use.**

Progress: The complete SNX1 coding region lies within 43kb of genomic DNA as defined by sequencing and oligonucleotide hybridization (Figure 2).

Task 4: **Month 8-12: Determine the complete sequence of the SNX1 gene.**

Progress: We have sequenced 15.7kb of the SNX1 gene. This represents 15 exons and considerable intronic sequence. Using this information and and an unordered working draft of sequences from human chormosome 15 submitted to GenBank by the Washington University genome Sequencing Center, we have generated a 99.2% sequenced 70.5kb map containing the human SNX1 structural gene between bp9860 and 53415. This completely defines the intron-exon structure of the SNX1 gene and the intron-exon boundaries (Figure 2).

Task 5: **Months 11-12: Prepare annual project report and a manuscript describing the human SNX1 gene.**

Progress: An annual report was prepared and submitted. A manuscript entitled "Structural and Functioanl Characterization of the Human Gene for Sorting Nexin 1 (SNX1) is in press in **DNA and Cell Biology**. This work was also presented in poster format at the Department of Defense Breast Cancer Research Progam "Era of Hope Meeting" in June 2000.

Technical Objective 2. Prepare a transgenic mouse line with SNX1 expression targeted to the mammary gland using the WAP promoter.

Task 6: **Months 7-9: Prepare the transgenic expression vectors.**

Progress: Transgenic expression vectors were prepared. The SNX1 genomic clone was too large for the proposed construction. Therefore we have adopted a cDNA approach that has been use successfully for stromolysin 1 (1) and for IGF (2). We obtained plasmid pbl103 containing bases -949 to +33 of the rat whey acidic protein (WAP) gene and plasmid pbTAPW3' containing 843 base pairs of WAP 3' sequence including the exons 3, 4 and the 3' untranslated region from Jeffrey Rosen, Baylor College of Medicine, Houston, Texas. We used these plasmids to generate a WAP-SNX1 cDNA vector. Our approach was to ligate the SNX1 cDNA into a WAP vector consisting of 943bp rat WAP 5' sequence and 675 bp WAP 3' sequence (Figure 3). To generate this construction, a 5' WAP PCR product was generated using oligonucleotides

5'-GATCGTCGACAAGGAGTATGGGCTGCACCA-3'

5'-GATCGAATTCGGCGGCGGCAGGCAAGTGAT-3'

as primers and pbl103 as the template. This PCR product contains rat WAP sequences -949 to -7. The 5' WAP PCR product was cleaved with SalI and EcoRI and cloned into the vector pBSIIKS(+) to generate pWAP 5'. Next, a 3' PCR product was generated using oligonucleotides

5'-GATCGAATTCAATGGCTGTATCATGAGTTG-3'

5'-GATCGCGGCCGCTCATTCTGTCAAGAGCTCAG-3'

as primers and pbTAPW3' as the template. The 3'WAP PCR product was cleaved with EcoRI and NotI and cloned into EcoRI/NotI cleaved pWAP 5' to generate pWAP 5' & 3' (Figure 3). The high fidelity polymerase Pfu was used for all PCR reactions reactions. An EcoRI fragment of SNX1 was cloned into the EcoRI site in pWAP 5' & 3'. The correct orientation of the SNX1 insert was determined by restriction enzyme mapping and nucleotide sequencing. The resultant plasmid, pWAP-SNX1cDNA was cleaved with SalI and NotI and the linearized minigene purified for mouse oocyte microinjection (Figure 4).

Task 7: **Months 10-13: Generate founder mice in collaboration with Jeffrey M. Rosen, Baylor College of Medicine.**

Progress: The pWAP-SNX1cDNA vector was constructed and a linearized DNA fragment was been purified and sent to the NICHD transgenic Mouse Development Facility at the University of Alabama at Birmingham for the production of transgenic mice. A total of 22 mice were born in three litters during the period December 2000 and March 2001. Of the 22 mice, 6 died prior to weaning.

Task 8: **Months 16-18: Identify transgenic lines, confirm SNX1 protein expression.**

Progress: A PCR assay was developed and validated for detecting the SNX1 transgene in FVB mouse tail blot DNA (figure 5). A graduate student, Anthony Eddington M.S., has been responsible for this aspect of the project. Of the 16 mice that survived to weaning, we have identified one transgene-positive female mouse in our PCR assay (figure 6). We will breed this mouse and screen for transgene inheritance. For analysis of protein expression, we generated two new antibodies were generated that work well in western immunoblotting experiments (figure 7). Laboratory stocks of an affinity-purified polyclonal anti-peptide antibody (#3904) that was to previously used to measure SNX1 protein expression were depleted.

Task 9: **Months 19-22: Determine the effect of overexpressing SNX1 in the mammary gland on the concentration of mammary gland EGF receptors**

Progress: We have initiated experiments to measure EGF receptor levels in saturation binding experiments. We have encountered batch to batch variability in the quality of commercial ^{125}I-EGF preparatios and are now iodinating our own EGF. In a separate project, we have analyzed several human breast tumor cell lines and use this to validate the ligand binding assay (figure 8). A technician, Ms. Susan Foreman, has been identified in a colleague's laboratory adjacent to my own to perform the assays. Susan has experience in more difficult measurements of β_2-adrenergic receptors in crude membranes and should have no problems performing the more sensitive EGF binding assays to measure EGF receptors in mouse mammary glands.

Task 10: **Months 23-24: Prepare annual project report and a manuscript describing the WAP-SNX1 mice and the consequences of SNX1 overexpression on EGF receptor levels in vivo.**

Progress: This annual report was been prepared. Preparation of a manuscript has not been initiated as that awaits the completion of tasks 8-9.

Technical Objective 3. Cross SNX1 mice with TGFα mice and measure mammary gland tumor incidence to determine if SNX1 can be used as a tumor suppressor.

Progress: The tasks in technical objective (task 11-14) cannot be begun until task 8 is completed. A 12 month no-cost extension of the expiration date of the grant was requested and has been granted.

KEY RESEARCH ACCOMPLISHMENTS

- Chromosomal Mapping of SNX1 and SNX2
- Characterization of the 60kb human SNX1 gene
- Analysis of alternative splicing of SNX1 in cDNA from human tissues
- Functional identification of the SNX1 promoter
- Completion of WAP-SNX1 minigene construction
- Validation of PCR assay to detect SNX1 minigenes in mouse tail DNA
- Generation and characterization of affinity-purified antibodies to detect the SNX1 protein

- 3 rounds of WAP-SNX1 minigene microinjeciton into oocyte yielding 22 mice
- Identification of 1 WAP-SNX1 minigene positive mouse

REPORTABLE OUTCOMES

Work completed for Technical Objective 1 was presented in poster format at the department of defense Breast Cancer Research Progam "Era of Hope Meeting" in June 2000. A mansucript was prepared and is in press in DNA and Cell Biology.

Work from my laboratory was presented in April 2000 at the American Association of Cancer Research meeting (Appendix 2). Although not directly related to the Statement of Work, the lead author, Cynthia Burroughs, Ph.D., was awarded a HBCU Faculty Scholar Research Award for this poster. Dr. Burroughs is on the faculty at a neighboring undergraduate institution, Philander Smith College, and has experience with mouse mammary glands. She works in the laboratory full-time during the summer and part-time during the academic year. This collaboration represents a Career Development effort that was especially relevant to procuring additional research support for the laboratory from the State of Arkansas Breast Cancer Research Program. Dr. Burroughs successfully competed for an award from this program and will assist in the planned animal experiments. In September 2000, I was appointed director for the Arkansas Cancer Research Center confocal microscope laboratory based on my expertise in digital light microscopy.

CONCLUSIONS

We have cloned and characterized the human gene for SNX1. The SNX1 gene is alternatively spliced and characterization of its structure will aid in understanding the regulation, and significance of the alternative splices. We have also identified the functional promoter in transfection experiments and find that it contains features characteristic of a house keeping gene. The SNX1 genomic clone and subclones that we now have in hand have also provided useful reagents for gene knockout studies to better understand the function of SNX1 inside cells. A graduate student, Parag Chowdhurry, has constructed a targeting vector and will attempt to use it to knockout the SNX1 gene in HEK 293 cells. Our determination of the chromosomal localization of SNX1 could facilitate the potential assignment of SNX1 as a disease locus identified in human genetic mapping studies. Currently, no diseases map to the SNX1 locus. Given the large size of the introns in the SNX1 gene, we decided not to pursue our first choice of replacing coding exons and intervening sequences of the whey acidic protein gene with SNX1 genomic sequences. Instead, we have inserted a SNX1 cDNA fragment to generate our minigene for injection into mouse oocytes.

Over the last year, other projects in my laboratory and in other laboratories (3-5) have led me to question the specificity and the utility of SNX1 in down-regulating EGF receptor expression. In part, these reservations may have arisen from our use of overexpression strategies to study SNX1. For this reason, transgenic mouse production was temporarily delayed while we evaluated the problem. As we proceeded with the transgenic mouse production efforts, we encountered substantial mortality in the pups, indicating that ectopic SNX1 overexpression might be lethal. Although we now have a transgene positive female, we do not yet know if SNX1 is expressed or if the transgene will be inherited.

We think that our plans for gene knockouts will provide additional support for the functional identification of SNX1 as a down regulator of EGF receptors using an experimental

approach distinct from overexpression. We now have evidence that SNX1 exists in a multi-protein complex consisting of oligomers of SNX1, SNX2 and possibly 3 additional proteins. The fact that SNX1 is part of a multi-protein complex may account for variability problems in our cultured cell assays. In any event, the conservation of function and biochemical properties between SNX1 and its yeast homologue, Vps5p (6), make it clear that SNX1 has a role in endosomal membrane trafficking. With our continued studies in cell culture models and transgenic animals, we expect to make considerable progress in understanding the role of SNX1 in cell proliferation and EGF receptor trafficking the mammary gland.

REFERENCES

1. Sympson CJ, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 125(3):681-93.

2. Neuenschwander S, Schwartz A, Wood TL, Roberts CT Jr, Henninghausen L, LeRoith D (1996). Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model.*J Clin Invest* 97(10):2225-32.

3. Haft, C. R., M. de la Luz Sierra, V. A. Barr, D. H. Haft, and S. I. Taylor. 1998. Identification of a family of sorting nexin molecules and characterization of their association with receptors. *Mol Cell Biol.* 18:7278-87.

4. Kil, S. J., M. Hobert, and C. Carlin. 1999. A leucine-based determinant in the epidermal growth factor receptor juxtamembrane domain is required for the efficient transport of ligand-receptor complexes to lysosomes. *J Biol Chem.* 274:3141-50.

5. Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W. Y. Langdon, L. Beguinot, B. Geiger, and Y. Yarden. 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* 12:3663-74.

6. Horazdovsky, B. F., B. A. Davies, M. N. Seaman, S. A. McLaughlin, S. Yoon, and S. D. Emr. 1997. A sorting nexin-1 homologue, Vps5p, forms a complex with Vps17p and is required for recycling the vacuolar protein-sorting receptor. *Mol Biol Cell.* 8:1529-41.

APPENDICES

Appendix 1 - Figure Legends

Figure 1. Chromosomal localization of SNX1 and SNX2. Fluorescence in situ hybridization of P1 genomic DNA clones isolated using SNX1 and SNX2 cDNA fragments. Biotinylated DNAs corresponding to genomic clones for SNX1 (3E2) and SNX2 (B100-3)

hybridized with efficiencies of 87.5% and 85%, respectively. Two additional P1 clones isolated with the SNX1 cDNA (6K1 and 6K7) yielded similar results.

Figure 2. Structural Organization of the SNX1 Gene and Correspondence to the SNX1 cDNA. The SNX1 gene map was generated using data generated in our laboratory and by the human genome sequencing project. The map is based on an insert containing 4.5 kb 5'-flanking DNA, a 43 kb 6K1 fragment that contained all the SNX1 coding DNA and approximately 16 kb 3' flanking DNA. The dark bars mark the locations of 15 identified exons. Below the genomic map is a cDNA map containing the SNX1 open reading frame within a 1974 bp cDNA. The location of a PX domain, (PX), several regions predicted to form coiled-coils (CC1, CC2, CC3) and a receptor binding domain (RDB) are superimposed on the open reading frame. The numbered boxes below the open reading frame indicate the regions of the cDNA encoded by the 15 exons. Several alternatively spliced (skipped) exons are designated Δ.

Figure 3. Map of plasmid pWAP 5'&3'. This plasmid was constructed by cloning PCR fragments corresponding to the 5' (WAP -949 to -7) and the 3'-end (WAP 3' PCR Product) of the rat WAP gene. The unique EcoRI site separating the WAP 5'-end and 3'-end fragments was used for insertion of the SNX1 cDNA.

Figure 4. Map of the plasmid from which the linearized SNX1 minigene is derived. Plasmid pWAP-SNX1cDNA was generated as described. The ethidium bromide stained agarose gel shows the 3375bp SalI-NotI linearized minigene fragment that was gel purified for use for microinjection into mouse oocytes to generate transgenic mice. The size was estimated by comparison to BstEII digested λ DNA. Also included on the agarose gel is a 4078bp WAP-GFP-SNX1 minigene that we generated for potential use in transgenic animal generation using the green fluorescent protein as a marker for SNX1 expression.

Figure 5. Specificity and sensitivity of pWAP-SNX1 transgene specific amplimers in the presence of FVB mouse tail genomic DNA. PCR reactions were performed in a volume of 25µl with the indicated amounts of pWAP-SNX1 transgene and/or FVB mouse genomic DNA. One primer was specific for human SNX1 and the other was specific for rat WAP DNA. Assuming that there are $1.6x10^5$ copies of transgene per 100ng genomic DNA, this assay will easily detect the specific transgene in mouse tail DNA.

Figure 6. Identification of a WAP-SNX1 minigene positive female mouse by PCR analysis of tail DNA. PCR reactions were performed in a volume of 50µl using FVB mouse genomic DNA samples and pWAP-SNX1 minigene-specific (A, B) or human SNX1-specific primers (C). The assay depicted in panel A identifies a single mouse (mouse 300) in a litter born March 19, 2001 that is positive for the microinjected pWAP-SNX1 minigene. However, the signal is very weak, and the positive control reaction is smeared. Panel B is a titration of the PCR reaction using the indicated number of copies of the minigene in the presence of FVB genomic DNA that demonstrates that the primers are working properly. To confirm that mouse 300 is positive for the WAP-SNX1 transgene, a different set of PCR primers were therefore used (C). Panel C is a PCR analysis of the same mouse genomic DNAs as in panel A using primers diagnostic for the human SNX1 gene. As was the case in panel A, mouse 300 is positive for the presence of human SNX1 DNA. Using this primer pair, the 5000 minigene copy control sample also yielded the expected product. Thus, mouse 300 is positive for the WAP-SNX1 minigene.

Figure 7. Western Blot Characterization of Affinity Purified Anti-SNX1 (Batch 2) using HeLa cytosols containing HA-SNX1. Cytosols were prepared from HeLa cells overexpressing a HA epitope-tagged SNX1. The indicated volumes of the cell extracts were

electrophoresed on SDS-PAGE gels, transferred to PVDF membranes and blotted with affinity purified antibodies raised against the peptide CKYLEAFLPEAKAIS. Antibody preparations from two different rabbits (5551/2 and 5552/2) were analyzed either fresh or following aliquoting and a cycle of freezing and thawing. From this analysis, we conclude that 5551/2 is the more sensitive and specific antibody preparation and that both preparations are stable to freeze-thaw.

Figure 8. Scatchard plots of saturation binding assays to measure EGF receptor numbers in cultured breast cell lines. Immortilized (HB2) and tumor cell lines (MCF7v and HBT22) derived from human breast tissues were cultured to 70% confluence and serum starved overnight. Cultures were chilled to 4C and incubated with ^{125}I-EGF in the absence or presence of increasing concentrations of mouse EGF for 4 hours. After washing, the cells were dissloved in 1N NaOH and the radioactivity measured in a gamma counter. The data were reduced by Scatchard analysis assuming a single class of ligand binding sites. Based on these measurements, we calculate that the MCF7v line (closed squares) expresses ~380,000 receptors per cell, the HB2 line (open squares) expresses ~77,000 receptors per cell and that the HBT22 line (triangles) is devoid of EGF receptors.

Figure 1

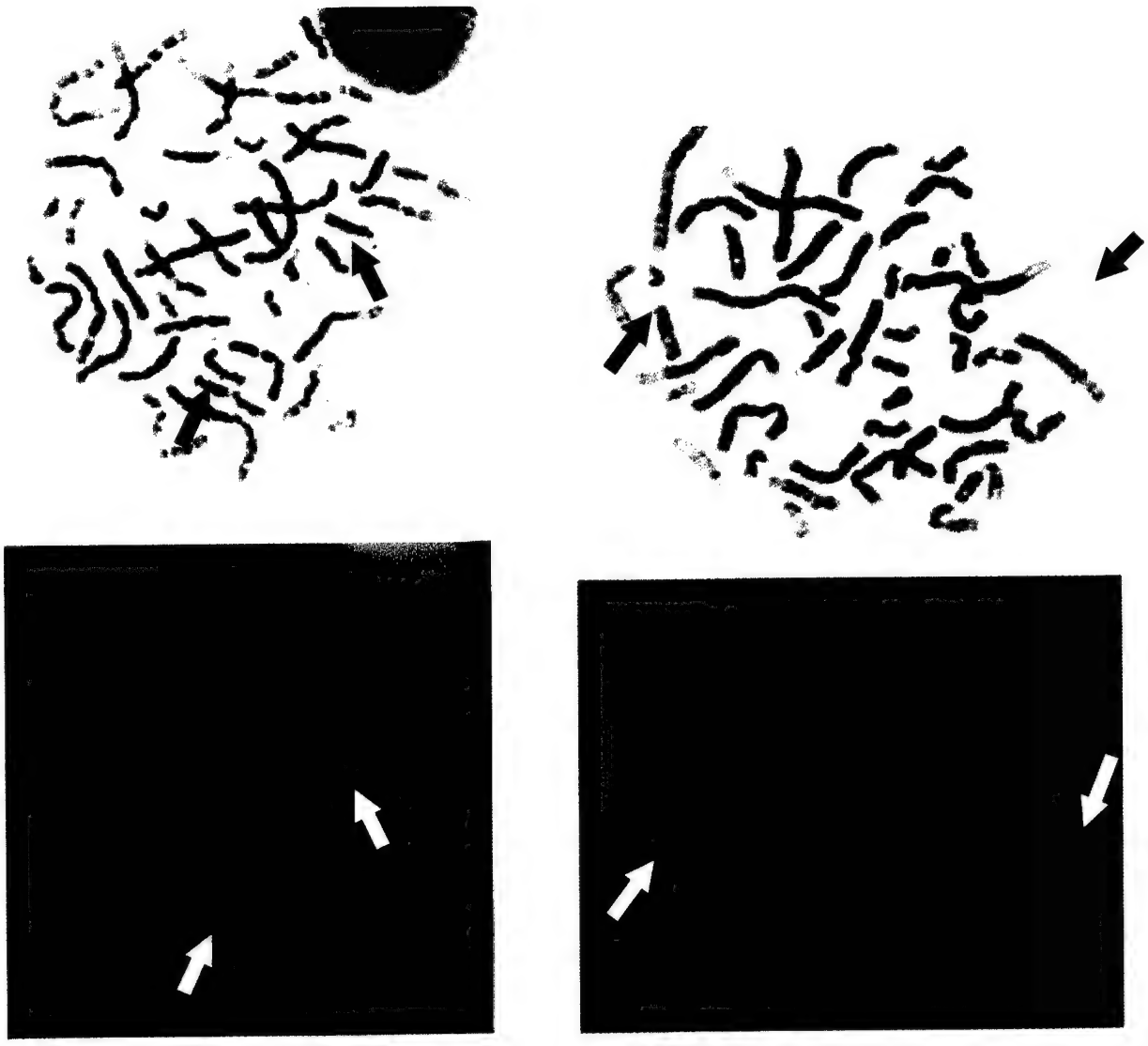


Figure 2

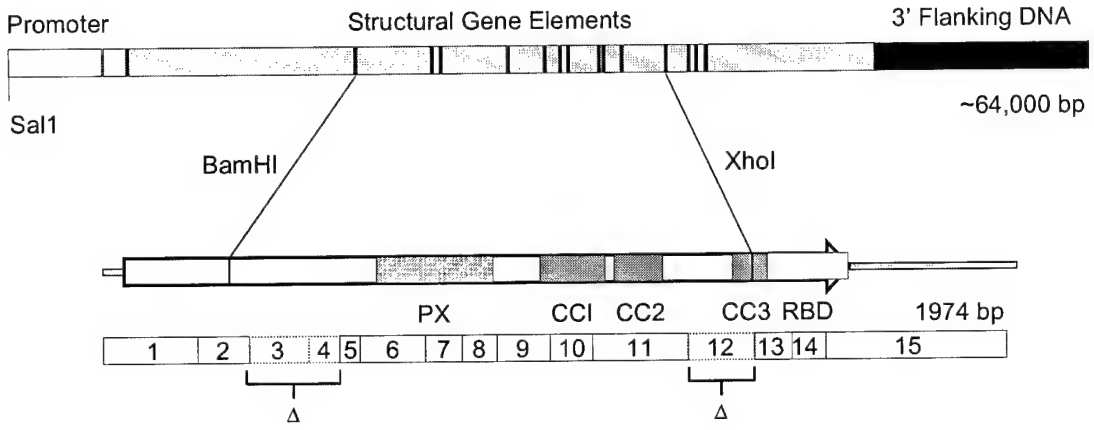

Promoter
Structural Gene Elements
3' Flanking DNA
~64,000 bp
Sal1
BamHI
XhoI
PX
CCI
CC2
CC3
RBD
1974 bp
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Δ
Δ

Figure 3

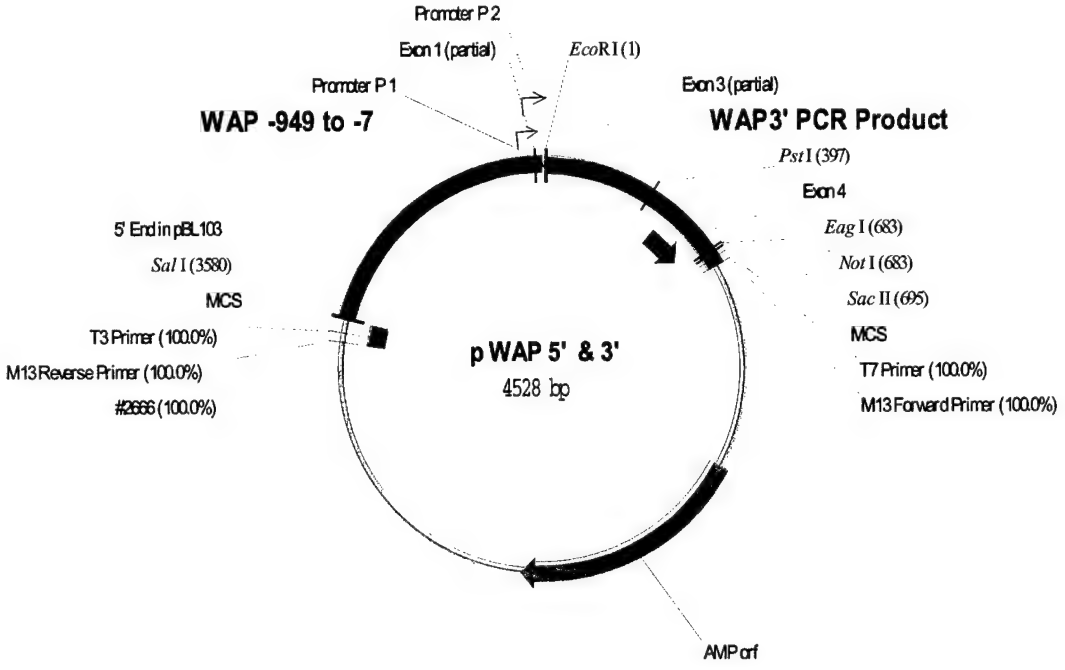
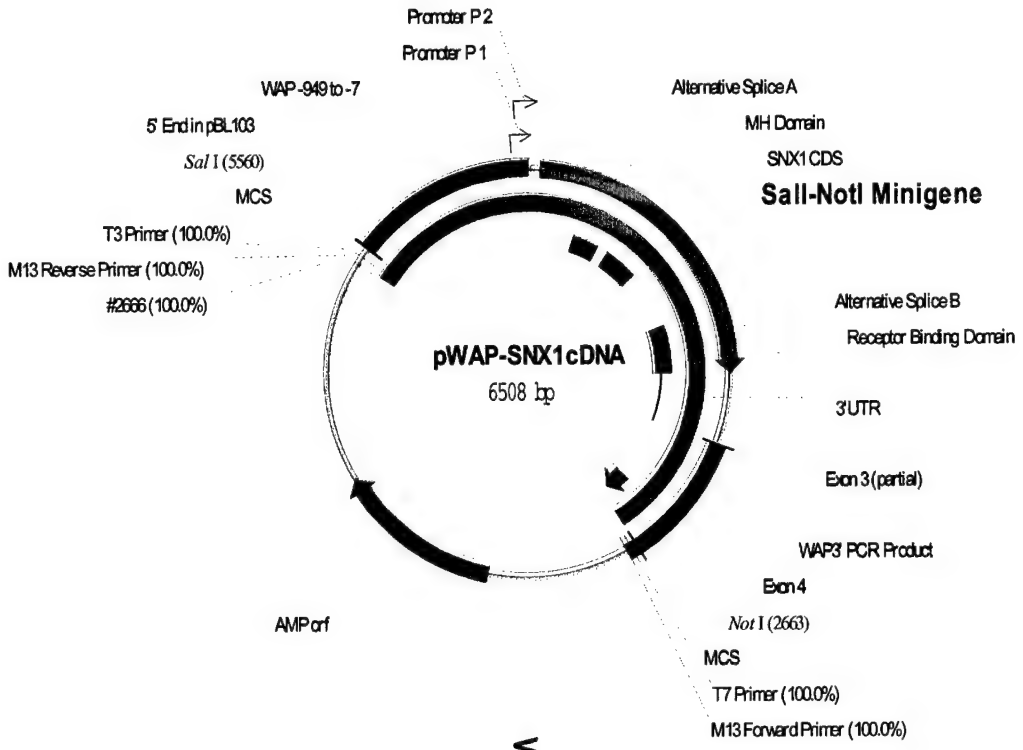

Promoter P2
Exon 1 (partial)
EcoR I (1)
Promoter P1
WAP -949 to -7
Exon 3 (partial)
WAP3' PCR Product
Pst I (397)
Exon 4
Eag I (683)
Not I (683)
Sac II (695)
MCS
T7 Primer (100.0%)
M13 Forward Primer (100.0%)
5' End in pBL103
Sal I (3580)
MCS
T3 Primer (100.0%)
M13 Reverse Primer (100.0%)
#2666 (100.0%)
p WAP 5' & 3'
4528 bp
AMP orf

Figure 4


Promoter P2
Promoter P1
WAP -949 to -7
5' End in pBL103
Sal I (5560)
MCS
T3 Primer (100.0%)
M13 Reverse Primer (100.0%)
#2666 (100.0%)
Alternative Splice A
MH Domain
SNX1 CDS
SalI-NotI Minigene
pWAP-SNX1cDNA
6508 bp
Alternative Splice B
Receptor Binding Domain
3'UTR
Exon 3 (partial)
WAP3' PCR Product
Exon 4
Not I (2663)
MCS
T7 Primer (100.0%)
M13 Forward Primer (100.0%)
AMP orf

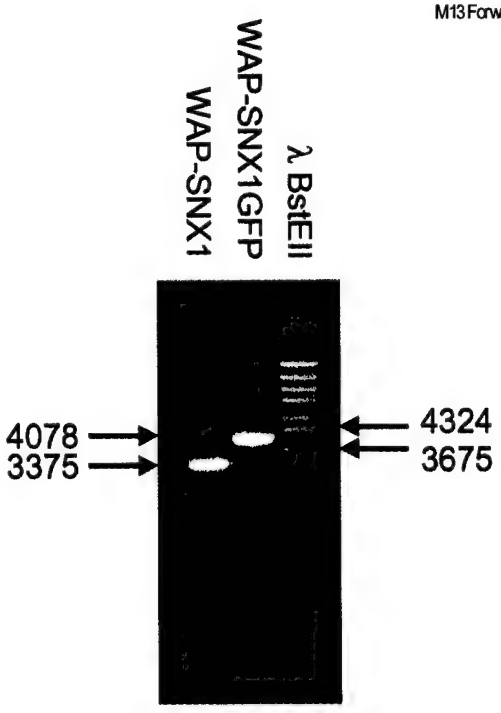

WAP-SNX1
WAP-SNX1GFP
λ BstEII
4078
3375
4324
3675

Figure 5

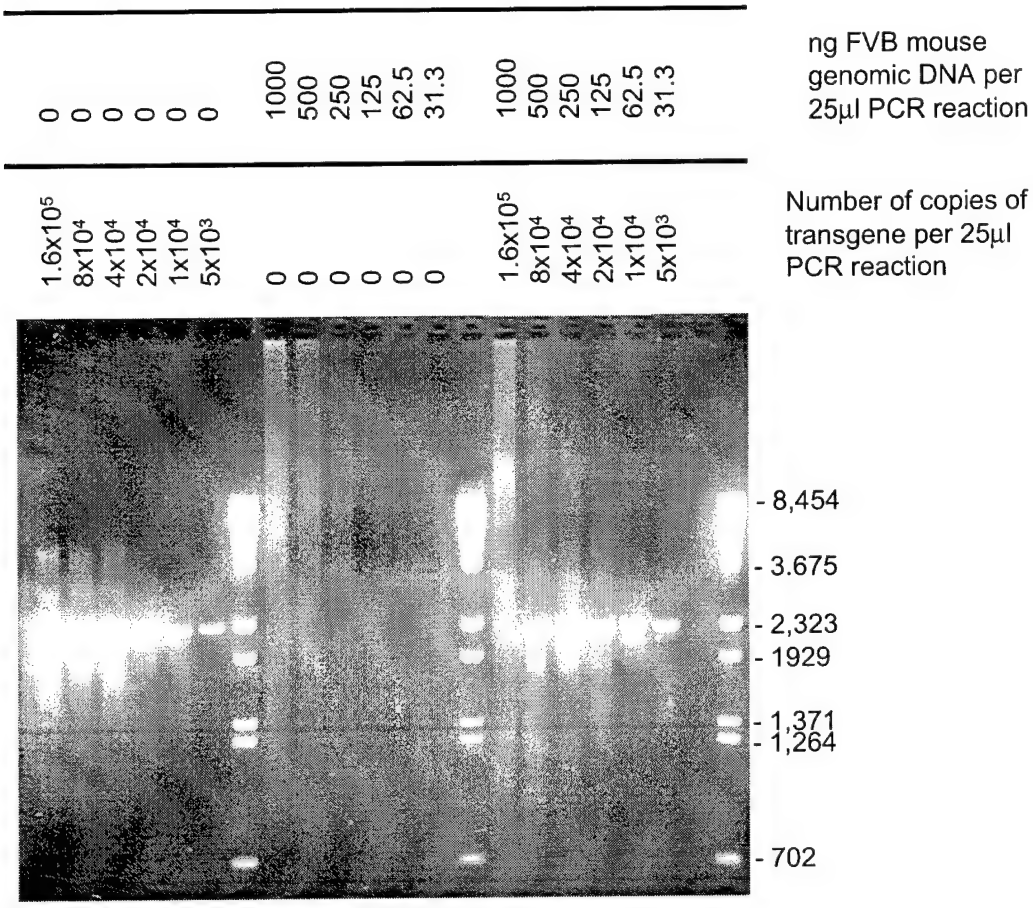

ng FVB mouse genomic DNA per 25µl PCR reaction
0
0
0
0
0
0
1000
500
250
125
62.5
31.3
1000
500
250
125
62.5
31.3
Number of copies of transgene per 25µl PCR reaction
1.6x10^5
8x10^4
4x10^4
2x10^4
1x10^4
5x10^3
0
0
0
0
0
0
1.6x10^5
8x10^4
4x10^4
2x10^4
1x10^4
5x10^3
- 8,454
- 3.675
- 2,323
- 1929
- 1,371
- 1,264
- 702

Figure 6

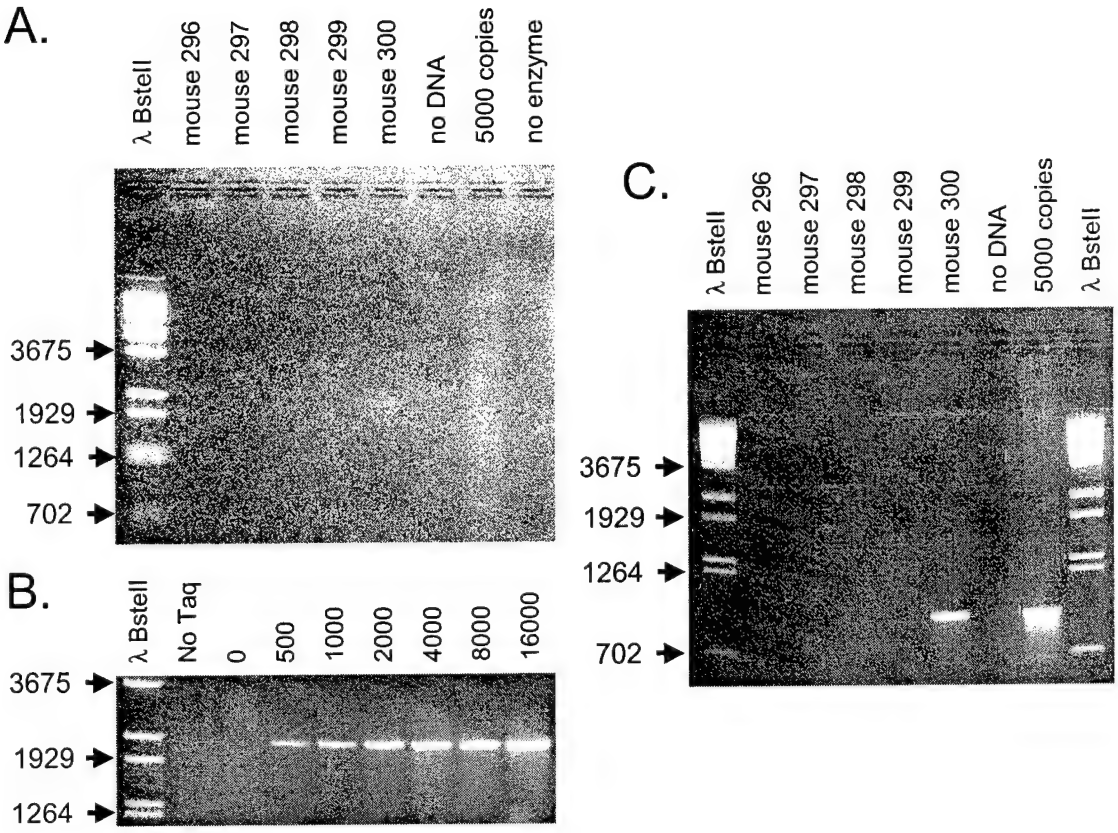

A.
λ BstEII
mouse 296
mouse 297
mouse 298
mouse 299
mouse 300
no DNA
5000 copies
no enzyme
3675
1929
1264
702
B.
λ BstEII
No Taq
0
500
1000
2000
4000
8000
16000
3675
1929
1264
C.
λ BstEII
mouse 296
mouse 297
mouse 298
mouse 299
mouse 300
no DNA
5000 copies
λ BstEII
3675
1929
1264
702

Figure 7

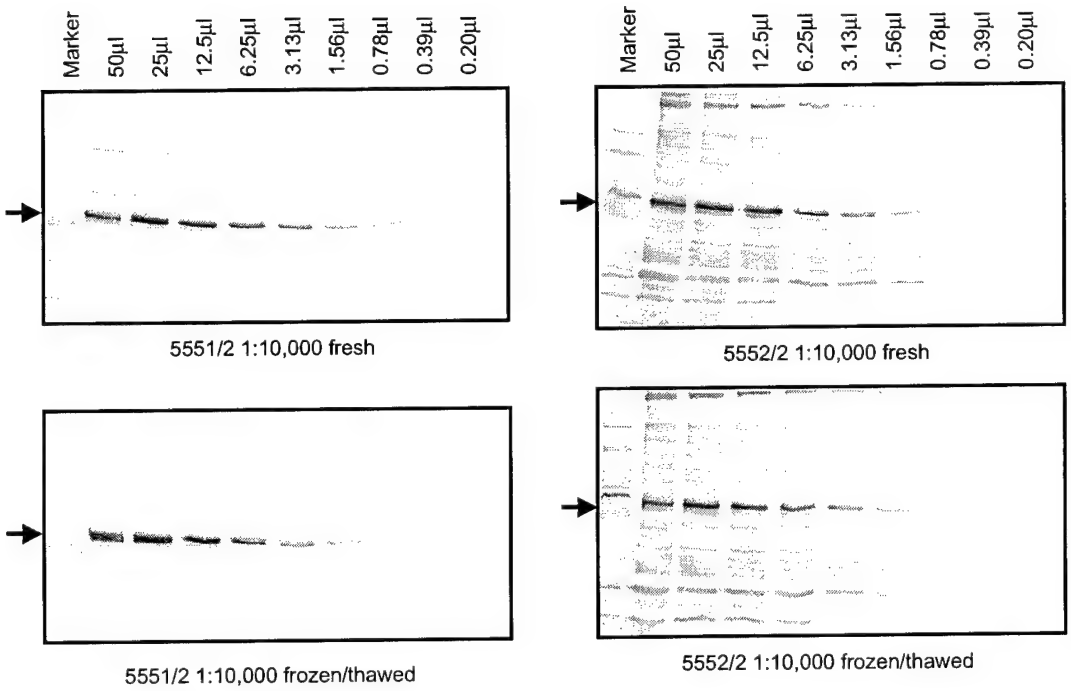
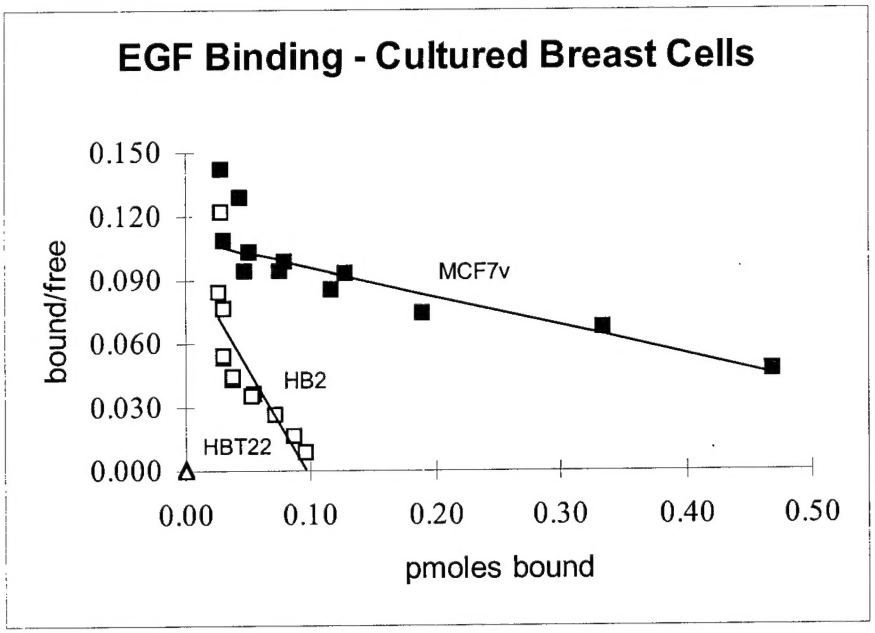

Marker
50µl
25µl
12.5µl
6.25µl
3.13µl
1.56µl
0.78µl
0.39µl
0.20µl
5551/2 1:10,000 fresh
5552/2 1:10,000 fresh
5551/2 1:10,000 frozen/thawed
5552/2 1:10,000 frozen/thawed

Figure 8


EGF Binding - Cultured Breast Cells
bound/free
0.150
0.120
0.090
0.060
0.030
0.000
MCF7v
HB2
HBT22
0.00
0.10
0.20
0.30
0.40
0.50
pmoles bound

Appendix 2 - Abstract of poster presentation

Poster presented at the DoD Breast Cancer research Program Era of Hope Meeting June 8-12, 2000 Atlanta, Ga.

Identification and Characterization of the Human SNX1 Gene. Richard C. Kurten, Bryan B. Shank and Anthony D. Eddington, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences

Excessive activation of growth factor receptors can lead to the unrestrained cellular proliferation characteristic of tumors. Our objective is to determine if SNX1, a protein involved in intracellular membrane trafficking, can be used to downregulate EGF receptors in mammary gland, thereby attenuating EFG receptor dependent proliferation. Our approach is to characterize the human gene for SNX1 and to generate transgenic mice overexpressing human SNX1 in the mammary glands. Three bacterial artificial chromosomes (BAC) containing the human SNX1 gene were isolated in collaboration with H. Steven Wiley (University of Utah School of Medicine). Fluorescence *in situ* hybridization was used to determine the chromosomal localization of SNX1 to human chromosome 15q22 and of SNX2 (a closely related protein) to chromosome 5q23. One of the BAC clones for SNX1, labeled 6K1, has been characterized by subcloning, restriction mapping and DNA sequencing. The complete SNX1 coding region lies within 55kb of genomic DNA as defined by sequencing and oligonucleotide hybridization and consists of at least 15 exons. Several of the intron/exon junctions correspond to sites for alternative splicing as previously defined in cDNA clones. Alternatively spliced SNX1 mRNAs were detected in a variety of tissues using reverse-transcriptase polymerase chain reactions but they were less abundant than the full-length SNX1 mRNA species. The first intron of SNX1 is 16.5 kilobases in length and therefore unsuitable for the production of a transgenic expression vector. We next adopted a cDNA approach that has been used successfully for stromolysin 1 (Sympson, et al., 1994, *J Cell Biol* 125(3):681-93) and IGF (Neuenschwander, et al. 1996, *J Clin Invest* 97(10):2225-32.). We obtained plasmid pbl103 containing bases -949 to +33 of the rat whey acidic protein (WAP) gene and plasmid pbTAPW3' containing 843 base pairs of WAP 3' sequence including the exons 3 and 4 and the 3' untranslated region from Jeffrey Rosen, Baylor College of Medicine, Houston, Texas. We used these plasmids to generate a WAP-SNX1 cDNA vector consisting of 943bp rat WAP 5' sequence and 820 bp WAP 3' sequence. pWAP-SNX1 was cleaved with SalI and NotI and the linearized minigene purified for mouse oocyte microinjection. To analyse for transgenics, we developed a set of amplimers and reaction conditions that detect 5000 copies of SNX1 transgene in the presence of 1.25 micrograms/ml FVB mouse tail genomic DNA. The production of transgenic animals with the vector we have prepared will allow us to test the utility of SNX1 for repressing EGF receptor dependent tumor growth in the mouse mammary gland.

The U.S. Army Medical Research and Materiel Command under DAPD17-98-1-8175 supported this work.

Appendix 3 - Manuscript in press

The attached manuscript is in press in DNA and Cell Biology.

Structural and Functional Characterization of the Human Gene for Sorting Nexin 1 (SNX1). Brian B. Shank, H.S. Wiley, and Richard C. Kurten

The aim of the present study was to identify the gene for SNX1 to evaluate the potential for tissue specific alternative splicing and to analyze the activity of the SNX1 promoter. The coding DNA for SNX1 is divided between 15 exons in 43 kb of genomic DNA located on human chromosome 15q22. Although SNX1 mRNA expression is widespread in human tissues, alternative splicing is thought to generate skipped exons in SNX1 cDNAs. Based on determination of the SNX1 gene structure and an analysis of SNX1 mRNAs in a variety of tissues using reverse-transcriptase polymerase chain reactions, we demonstrate that SNX1 mRNAs are alternativelly spliced. Exon skipped products were less abundant than full-length SNX1 mRNA species, but the ratio of skipped to full-length mRNA indicated that alternative splicing may be developmentally regulated in liver. Consistent with widespread mRNA expression, the SNX1 promoter was GC rich and lacked a TATA box, features characteristic of housekeeping promoters. SNX1 promoter activity was dependent on the presence of proximal promoter sequences that contain initiator elements and predicted binding sites for the transcription factors Sp1 and E2F. These findings indicate that regulation of SNX1 gene expression at the transcriptional level is likely minor. Rather, developmentally specific exon skipping provides a potential mechanism for regulating the activity of SNX1.

Appendix 4 - Abstract resulting in award for the first author

Poster presented at the 91st Annual Meeting of the American Association of Cancer Research April 1-5, 2000 San Francisco, CA.

INTRACELLULAR POOLS OF RECEPTORS MODULATE THE KINETICS OF RECEPTOR DOWNREGULATION. C. Burroughs, R. Smith, M. McIntire and R. Kurten. University of Arkansas at Pine Bluff, Pine Bluff, AR 70611; University of Arkansas School for Medical Sciences, Little Rock, AR 72205

Different distributions of erbB2 receptors have been documented in different cell lines. In some cell lines, most receptors are inside the cell whereas in others most of the receptor is external. We have examine the distribution of epidermal growth factor receptor (EGFR) and the related receptor erbB2 in a virus transformed cell line derived from normal breast tissue (HB2), in an MCF7 cell line, and in a variant cell line derived from MCF7. Each breast cell line was grown to 70% confluence in DMEM/F12 with 10% bovine calf serum and 1% antibiotics. Cells where washed with PBS and serum starved for 24hr in DMEM/F12, 0.01% BSA and 1% antibiotics. Cells were exposed to 100 nM EGF in DMEM/F12, 0.01% BSA and 1% antibiotics and terminated at 2,4,6,12 and 24 hours after treatment. EGF receptor and erbB2 expression was examined in detergent extracts by western blotting. HB2 and MCF-7 cells expressed modest amounts of both EGF and erbB2 receptors. Treatment with EGF for 2 hours efficiently down-regulated both EGF and erbB2 receptors. By contrast, the variant MCF7 cells expressed high levels of receptors and the total mass of receptors was not affected by treatment with EGF for 2 hours. However, when variant MCF7cells were treated for 12 or 24 hours, there was efficient down-regulation of both EGF receptor and erbB2.